Microbubbles in molecular imaging and therapy

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Microbubbles and acoustically active materials can be applied as targeted contrast agents.

Microbubbles and acoustically active materials can be applied as targered diagnostic agenrs for molecular imaging with ultrasound, and in other novel hybrid imaging recnniques. These materials can also be used as therapeutic agents for rrearing thrombosis and vascular plaques, and for drug and gene delivery, offering the potential to integrate diagnostic imaging and therapy into a new paradigm.

Imaging and therapy

Ultrasound is extremely sensitive to the presence of microbubbles.

Ultrasound is extremely sensitive to the presence of microbubbles. In fact, cavitation imaging and other techniques are capable of detecting a single microbubble. On a molecular basis, in terms of sensitivity to a small number of atoms of a contrast agent, ultrasound rivals nuclear medicine and optical imaging, and exceeds MRI by several orders of magnitude. It exceeds computed tomography and standard X-ray techniques by an even greater amount.

Definity® contrast agent

Definity® is an FDA-approved ultrasound contrast agent, developed by ImaRx, and marketed in the US and Canada by Bristol Meyers Squibb for cardiac imaging. In Canada it is also marketed for radiological imaging. Definity contains perfluoropropane/air-filled phospholipid coated microbubbles, and exemplifies the sensitivity of ultrasound to minute amounts of ultrasound contrast.

Intravenous bolus doses of 100 microliters (0.1 ml) of Definity are sufficient ro provide strong contrast, while, on the other hand, MRI might require a bolus dose of 40 cc of a gadolinium agenr, i.e. more rhan 400 times as much. In molecular imaging, a small concenrration of microbubbles provides sufficient contrast.

Definity microbubbles have a mean size of about 1–2 microns, with appreciable quantities of microbubbles well below a micron in size. For ultrasound imaging, the microbubbles are acoustically reflective over a range of frequencies from below 1 MHz to above 20 MHz. Definity is a blood pool agent. It is generally not appreciably taken up by macrophages, but recirculates in the blood, traveling throughout the vascular system in a similar way to red blood cells, until an appreciable quantity of gas has cleared across the membrane. Fluorocarbon gases are then cleared from the blood by the lungs.

While Definity is not considered to be the prototypical agent for molecular imaging, it nevertheless has important applications in molecular imaging, particularly for imaging angiogenesis. Definity also serves as a platform for the development of other targeted molecular imaging agents.

Angiogenesis

Angiogenesis, the process by which new blood vessels are developed, is seen in disease processes such as cancer and inflammation. It can be exploited therapeutically as a drug target. For example, angiogenesis can be stimulated to treat ischemic vascular and heart disease by improving collateral flow. Angiogenesis is also the target for new drugs, aimed at stopping the formation of new blood vessels, for the treatment of cancer, inflammation and retinopathy. In angiogenesis, blood flow increases, and the neovasculature generally has different architecture and hemodynamic properties from vessels in tissues not affected by angiogenesis. Ultrasound and ultrasound blood pool contrast agenrs such as Definity can be used to detect and porentially quantify angiogenesis.

To image angiogenesis with a blood pool agent such as Definity, the contrast agent is administered inravenously, and images can be obtained with a number of different contrast-specific ultrasound pulse sequences. Such pulse sequences decrease the signal from background tissues and increase the signal from rhe conrrast agent. Contrast-enhanced ultrasound can then clearly show the neovasculature of angiogenesis. Microbubbles may be destroyed

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using a higher energy ultrasound pulse, i.e. one with an increased Mechanical Index (MI). This can be used to create a square bolus arriving, for example, at the microvascular bed in a tumor. Using high-MI pulses to destroy the contrast, and then following re-entry of contrast with low-MI imaging, makes it possible to measure the rate of inflow of contrast. The rate of inflow at which perfusion reappears corresponds directly to tissue perfusion. Perfusion can then be quantified at the microvascular level. The images can be evaluated for rate of flow, perfusion, vascular architecture and morphology. Comparison of serial studies in patients under treatment with angiogenesis inhibitors (also potentially chemotherapeutics) are likely to be useful for following the results of treatment, monitoring and modifying therapy.

Currently many follow-up patients, particularly cancer patients, are studied with CT and MRI in order to assess the response to therapy. Generally, response is judged by changes in bi-dimensional measurements of tumor diameters. However, changes in angiogenesis will precede changes in tumor size.

Rapid tests based on contrast-enhanced ultrasound using blood pool agents may enable more rapid assessment of response to therapies based on chemotherapy, angiogenesis inhibitors and other drugs. Favorable response to a selected therapy should be seen on angiogenesis imaging as a reduction in neovasculature formation, prior to changes in bi-dimensional tumor measurements. Development of contrast-enhanced angiogenesis imaging with ultrasound will make a cost-effective method for detecting disease and monitoring response to therapy.

Targeted contrast agents

In addition to using ultrasound contrast agents for imaging blood flow, it is also possible to make targeted contrast agents that bind to selected cells, providing a more precise method of molecular imaging. Living cells can then be used as carriers for contrast agents. In this case the cells themselves can be exploited to target certain disease processes.

Leukocytes and lymphocytes travel throughout the body, and migrate to sites of inflammation. The function of leukocytes and other phagocytic cells is to engulf 'foreign' particles, and they will therefore engulf the microbubbles. Phagocytosis of the microbubbles can be enhanced by incorporating certain materials, e.g. phosphatidylserine, into the coating on the microbubbles. Such agents targeting white blood cells can be used to image inflammation. Lindner has shown that white blood cells carrying microbubbles will enhance abscesses and ischemic lesions following reperfusion [1–3].

Contrast-enhanced ultrasound clearly shows the neovasculature of angiogenesis.

Precise molecular imaging with ultrasound contrast agents may also be achieved by incorporating targeting ligands into the surface of the microbubble. The targeting ligands may be in the form of modifications in the coating material stabilizing the microbubbles, or specific molecules attached to the surface of the microbubbles. In our group we have developed a number of different targeted microbubble preparations for molecular imaging. Broadly speaking, these comprise agents for targeting the vasculature, and agents for targeting structures that lie beyond the vasculature.

For targets within the vasculature, micron-sized microbubbles are probably adequate. For targets beyond the vasculature, it is generally best for the particles to be small, certainly less than 500 nm diameter, and preferably much smaller. Blood vessels are lined by endothelial cells which have fenestrations that generally permit passage of molecules and very small particles from the intravascular into the extravascular space. In the brain, however, there are tight junctions between these endothelial cells, creating the blood brain barrier (BBB).

The BBB prevents passage of most molecules and particles into the central nervous system (CNS). Outside the CNS, however, the fenestrations of the endothelial barrier allow passage of very small particles, and this can be exploited for molecular imaging. Nanoparticles bearing targeting ligands can then be used for targeted molecular imaging and therapy.

It is possible to make targeted contrast agents that bind to living cells.

While microbubbles typically have diameters of the order of 1 to 2 microns, and are sometimes as small as a few hundred nanometers, other materials, e.g. perfluorocarbon (PFC) emulsions, can be much smaller in diameter: down to the range of 100 nm diameter, and potentially even smaller.

Microbubbles are mainly suitable for targeting vascular targets that are expressed on endothelial cells or on other cells exposed to circulating blood.

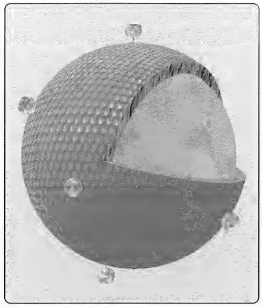


Figure 1.
Artist's impression of a targeted microbubble: a gas microbubble is covered in a lipid membrane in which targeting ligands have been incorporated.

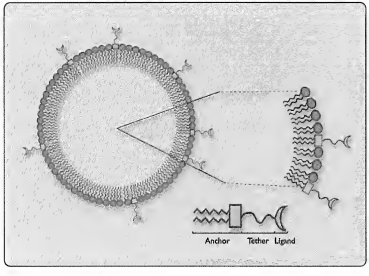


Figure 2.
Microbubble with bioconjugates attached. The enlarged view shows the anchor, tether and ligand. Adapted with permission from: Unger EC et al. *Therapeutic Applications of Microbubbles.* Eur J of Rad 2002; 42: 160–168.

A size smaller than several hundred nanometers diameter (e.g. perfluorocarbon droplets) is desirable if the particles are to extravasate from the vasculature to target cell surface epitopes on epithelial cells and others. This can be achieved with nanobubbles and PFC emulsions.

Many important molecular targets are expressed within the confines of the vascular system. Such targets include a variety of integrins such as GPIIBIIIA (expressed on activated platelets in thrombosis), AlphaVBetaIII (an important integrin expressed in angiogenesis associated with cancer and inflammation) and others. The selectins, e.g. p-selectin, are important targets involved in leukocyte rolling and inflammation.

Examples

Targeted microbubbles

Figure 1 depicts a targeted microbubble. The microbubble has bioconjugates incorporated into the membrane stabilizing the microbubble. Figure 2 shows how the bioconjugates may be built and stabilized within the membrane. We have worked on a number of peptides and other molecules as targeting ligands incorporated into bioconjugates. The bioconjugate has a polyethyleneglycol tether between the ligand and the lipid anchor inserted into the microbubble. MRX-408 is a recently developed microbubble incorporating bioconjugates targeted to the GPIIBIIIA receptor of activated platelets.

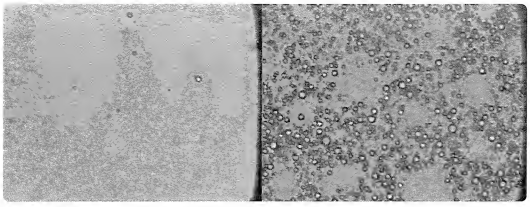
Intravascular applications

Figure 3 shows how MRX-408 microbubbles bind to a blood clot, while non-targeted microbubbles do not. Figure 4 shows in-vivo imaging of thrombosis in the left atrial appendage of a dog. The targeted microbubbles enhance the clot and improve the ability to detect the clot in the left atrial appendage. This could be clinically relevant.

Thrombosis in the left atrial appendage of patients with atrial fibrillation is an important clinical problem. [4–7] Such clots can break off and embolize to the brain, causing stroke. They are difficult to detect. Thrombus-targeted microbubbles have the potential to improve detection of left atrial thrombosis before it causes stroke.

Angiogenesis

In addition to thrombosis, there are other intravascular targets for molecular imaging with ultrasound. Certain integrins such as AlphaVBetaIII are expressed in angiogenesis. Lindner and others [8] have shown that microbubbles targeted to AlphaVBetaIII can be used to measure the temporal expression of this integrin in association with angiogenesis. A contrast agent targeted to endothelial-based markers of angiogenesis might be used to improve diagnosis and treatment of disorders affected by angiogenesis. Other endothelial-based



▲ Figure 3.
Photomicrographs of microbubble binding assay. The left frame is a non-targeted control sample. The scarcity of bubbles shows the lack of non-specific binding. The right frame contains targeted microbubbles and shows a great deal of binding. The concentration of non-targeted microbubbles was, in fact, much higher than that of targeted microbubbles. Reprinted with permission from: Unger EC et al. *Therapeutic Applications of Microbubbles.* Eur J of Rad 2002; 42: 160–168.

targets such as P-selectin might be exploited to develop targeted imaging agents for detecting inflammation.

Vulnerable plaque

Another important intravascular target is vulnerable plaque. Vulnerable plaques are those that have been infiltrated by macrophages, and are undergoing inflammation [9]. Inflammation can lead to rupture of vulnerable plaque and formation of thrombus, as in stroke and myocardial infarct.

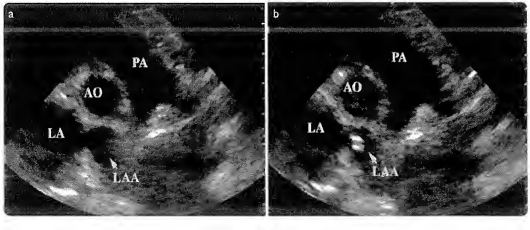
Vulnerable plaques may lie hidden as unseen threats, liable to cause morbidity and sudden death. A non-invasive test is needed to detect vulnerable plaque. Vulnerable plaque has been successfully detected using targeted microbubbles in combination with ultrasound [10].

Extravascular applications

For targeting beyond the vasculature, the particles usually need to have very small diameters. This requirement can be met by nanobubbles and perfluorocarbon emulsions. Targeting ligands can be incorporated into these systems as described above. The ligands may then be directed to targets on the surface of cells or in the intercellular matrix outside the vasculature. Very small bubbles (i.e. those well below 500 nm diameter) can be detected with high-frequency imaging and other ultrasound techniques. When sufficient quantities of these structures are delivered to a tissue, they can act as specular reflectors and greatly increase the backscattered signal. Smaller quantities can be detected with cavitation imaging. Nanobubbles may make it possible to target, for example,

For targeting beyond the vasculature, the particles must have very small diameters.

▼ Figure 4.
Ultrasound image identifying atrial appendage clots before (left) and after (right) administration of targeted microbubbles. PA = pulmonary artery, AO = aorta, LA = left atrium, LAA = left atrial appendage. Reprinted with permission from: Unger EC et al. in *Ultrosound Contrast Agents 2nd Edition* (Chapter 31). Editors: Goldberg BB, Raichlen JS, Forsberg F. Martin Dunitz Ltd. 2001.



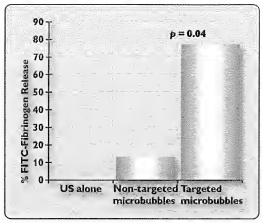


Figure 5.
In this in-vitro study, clots were exposed to 1.1 MHz focused ultrasound for 10 minutes. Ultrasound alone had no effect on clot lysis. MRX-815 non-targeted microbubbles significantly increase clot lysis. At the same concentration, the targeted microbubbles improve lysis sixfold compared with the non-targeted microbubbles.

epithelial cells in carcinoma. Such agents might be used to not only detect but also to treat diseases such as cancer.

Therapy

Ultrasound contrast agents afford unique potential as targeted molecular agents for therapy. Apfel and others [11] have shown that microbubbles lower the energy threshold for cavitation. When microbubbles cavitate, they concentrate the ultrasound energy within a certain region. This region can be selected by steering and focusing the ultrasound beam. Targeted molecular imaging ultrasound agents can be used as beacons to detect regions of disease and then to concentrate ultrasound energy within the target region. Contrast enhanced ultra-

Figure 6.
Ultrasound image demonstrating vessel patency after infusion of MRX-CS1 and exposure to ultrasound in a rabbit model. Reprinted with permission from: Unger EC et al. in *Ultrasound Contrast Agents 2nd Edition* (Chapter 31). Editors: Goldberg BB, Raichlen JS, Forsberg F. Martin Dunitz Ltd. 2001.

Figure 6a.
Baseline ultrasound image of femoral artery.

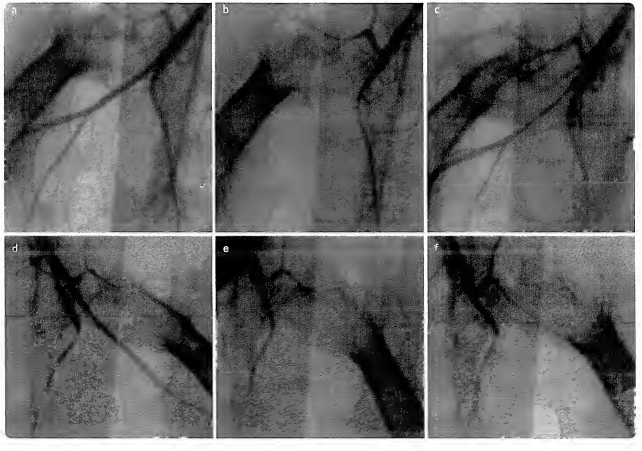
Figure 6b.
Ultrasound image after occlusion of femoral artery.

Figure 6c.
Ultrasound image after intravenous infusion of MRX-CS-1 and 15 minute exposure to ultrasound. (Note patency of femoral artery).

Figure 6d.
Baseline ultrasound image of the contralateral femoral artery.

Figure 6e.
Ultrasound image after occlusion of femoral artery.

Figure 6f.
Ultrasound image after intravenous infusion of MRX-CS1 without ultrasound.



sound has applications for treatment of thrombosis and drug delivery.

Sonothrombolysis

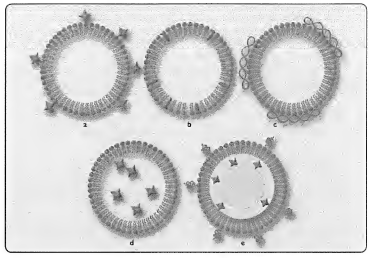
Figure 5 shows the results from sonothrombolysis (SonoLysis®) treatment of thrombus in vitro. Microbubbles accelerate the rate of SonoLysis. Enhancement is greater for the targeted molecular ultrasound contrast agent, MRX-408, than for the non-targeted agent, Definity. Concentration of the cavitation nuclei into the clot via receptor-mediated interaction provides more efficient transfer of the energy from cavitation to the thrombus than in the case of non-targeted microbubbles. Microbubble-enhanced SonoLysis may have potential clinical applications for rapid and safe treatment of vascular thrombosis. This could have clinical applications for treating myocardial infarction, stroke and deep venous thrombosis. Figure 6 shows images of in-vivo SonoLysis in a rabbit. Occlusive thrombus was created in the animal's femoral artery. SonoLysis using ultrasound at 200 kilohertz with microbubbles restored blood flow. Ultrasound without microbubbles was unsuccessful in restoring blood flow.

Passing the blood brain barrier

Hynynen has shown [12] that intravenous doses of microbubbles and transcranial application of ultrasound can be used to reversibly open the blood brain barrier. This can be exploited to deliver drugs to the CNS. As the BBB is opened, co-administered drugs may then passively enter the brain. Potentially, molecular targeted agents with ultrasound activation might be used to afford precise entry into the CNS by controlling the BBB at the molecular level.

Targeted drug delivery

As shown in Figure 7, drugs can be incorporated into microbubbles, nanobubbles and perfluorocarbon emulsion by a variety of different methods. Targeted drug delivery with this technology is probably most useful for highly active drugs that do not require large payloads of drug for biological effect. Many chemotherapeutics, proteins, gene-based drugs and other therapeutic agents are sufficiently active for delivery with ultrasound and targeted acoustically active carriers.



▲ Figure 7.
Different ways microbubbles can transport drugs. In these examples the stabilizing materials are shown as lipids, but they could also be polymeric materials. Adapted with permission from: Unger EC et al. in *Ultrasound Contrast Agents 2nd Edition* (Chapter 31). Editors: Goldberg BB, Raichlen JS, Forsberg F. Martin Dunitz Ltd. 2001.

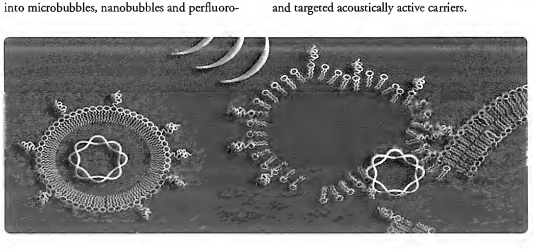
Figure 7a.
Drug attached to the membrane surrounding the microbubble.

Figure 7b.
Drug imbedded within the membrane itself.

Figure 7c.
Material such as DNA bound noncovalently to the surface of the microbubble.

Figure 7d.
Drug and gas loaded into the interior of the microbubble.

Figure 7e.
Hydrophobic drugs can be incorporated into a layer of oily material that forms a film around the microbubble, which is then surrounded by a stabilizing membrane. In this example a targeting ligand is incorporated in the membrane, allowing targeted delivery of the drug.



▲ Figure 8.
Gene delivery using ultrasound. The presence of gas in the gene-filled microbubbles allows ultrasound energy to burst them. An energetic wave is created, allowing the genetic material to enter the cells. Adapted with permission from: Unger EC et al. *Therapeutic Applications of Microbubbles.* Eur J of Rad 2002; 42: 160–168.

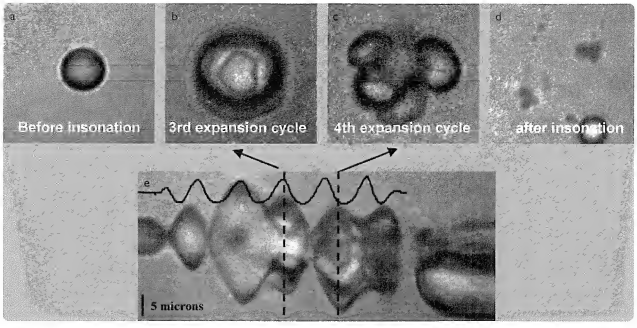


Figure 9. ▲
Optical images of a triacetin-shelled AAL with 2D frames acquired at times indicated on the streak image. AAL was insonated with a five-cycle pulse with a peak negative pressure of 3.0 Mpa, at 1.5 MHz.

Figure 9a. Before insonation, radius is 4.5 µm.

Figure 9b. 2D image acquired during the third expansion cycle.

Figure 9c. 2D image acquired during the fourth expansion cycle.

Figure 9d. 2D image acquired after insonation and subsequent fragmentation.

Figure 9e.
Streak image. The horizontal axis is time, with the entire image representing 5 µs. The vertical axis indicates radial distance taken through the center of the AAL. Reprinted with permission from May DJ et al. *Dynamics and Fragmentation of Thick-shelled Microbubbles.* IEEE Oct 2000; 49,10: 1400–1410.

Carriers bearing targeted ligands can be used for molecular imaging and therapy.

Figure 8 shows a microbubble binding DNA undergoing cavitation induced by ultrasound. As the microbubble cavitates, the DNA is accelerated into a target cell. Microbubble-based gene delivery agents can be monitored by ultrasound and activated with acoustic energy for more effective gene delivery. Targeted, acoustically active gene carrying agents have enhanced properties for gene delivery.

Figure 9 shows the ultrasound response of acoustically active lipospheres (AALs) [13–14]. The time-registered 'streak' image shows five oscillations of AALs in response to five pulses of ultrasound. The fourth image of the series shows fragmentation of the AAL post insonation. Nanodroplets have cavitated from the AAL. The same process can be used to deliver drugs in vivo. AALs and other acoustically active carriers can be formulated to bind therapeutic doses of drugs such as paclitaxel. As these carriers enter the region of the ultrasound field they can detected and activated with ultrasound energy. As the drug-carrying bubbles cavitate they create a microscopic shock wave and increase the permeability of the blood vessels. Material can be driven ballistically from the vessel, e.g. a capillary, into the surrounding tissue. We refer to this as SonoRelease®. SonoRelease can be used to deliver high concentrations of drug to selected tissues.

Carriers bearing targeting ligands can be used as molecular imaging and therapeutic agents. With a molecular approach, drug delivery is even more localized and controlled, and potentially more effective.

Figure 10 shows gene expression in vitro in cells expressing the receptor for fibroblast growth factor (FGF2). FGF2-targeted perfluorocarbon gene carriers (FluoroGene®) have enhanced efficiency for transfection. Targeted molecular agents can be monitored and activated by ultrasound to give localized gene delivery and therapy.

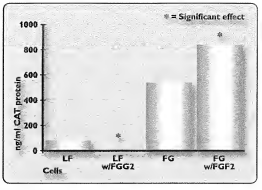
Future potential

Microbubbles and ultrasound have the potential to be incorporated into other new paradigms for molecular imaging. For example, magnetically responsive materials can be incorporated into micro-

bubbles for MRI, and optically active materials for optoacoustic imaging. Hybrid forms of imaging incorporating magnetic and optical properties with microbubbles may be exploited to increase sensitivity and therapeutic potential. Such hybrid imaging techniques may improve sensitivity for molecular imaging and our ability to characterize disease.

Ultrasound, a widely dispersed technology for medical imaging, has enormous potential for molecular imaging. The high sensitivity to microbubble agents and our ability to develop targeted molecular microbubble/nanobubble agents opens a new frontier in molecular imaging.

The ability to exploit cavitation opens potential for SonoLysis and drug delivery to the brain. Development of acoustically active targeted carrier systems for drugs opens potential for SonoRelease of drugs and genes at the molecular level.



▲ Figure 10.
A modified lipid, containing fibroblast growth factor (FGF2) as a targeting ligand, was added to the lipid shell of a liposome composed of a commercially available cationic lipid (lipofectamine) and the FluoroGene particle. These targeted FluoroGene particles were used to deliver a marker gene to cultured kidney cells expressing the receptor for FGF2. The expression level of the marker gene was significantly higher with the targeted FluoroGene particle than with either of the commercial preparations or the non-targeted particles. (LF = Lipofectamine, FGF2 = fibroblast growth factor, FG = FluoroGene).

Targeted micro/nanobubbles open a new frontier in molecular imaging.

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